Detection of *Gallibacterium* spp. in Chickens by Fluorescent 16S rRNA In Situ Hybridization

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Received 18 April 2003/Returned for modification 27 July 2003/Accepted 11 August 2003

Gallibacterium has recently been included as a new genus of the family *Pasteurellaceae* Pohl 1981, which encompasses bacteria previously reported as *Pasteurella anatis*, “*Actinobacillus salpingitidis*,” and avian *Pasteurella haemolytica*-like organisms. So far, identification has exclusively relied on phenotypic characterization. We present a method based on a cyanine dye 3.18-labeled in situ hybridization probe targeting 16S rRNA to allow specific detection of bacteria belonging to the genus *Gallibacterium*. The probe, GAN850, showed no cross-reactivity to 25 other poultry-associated bacterial species, including members of the families *Pasteurellaceae*, *Enterobacteriaceae*, and *Flavobacteriaceae*, when cross-reactivities were evaluated by whole-cell hybridization. The probe was further evaluated by hybridization to formalin-fixed spleen and liver tissues from experimentally infected chickens, in which it proved to be useful for the detection of *Gallibacterium*. Additionally, determination of the spatial distribution and the host cell affiliation of *Gallibacterium* at various times during the infection process was possible. In conclusion, the in situ hybridization technique described may be of use as a diagnostic tool as well as for studies to elucidate the pathogenesis of *Gallibacterium* infections in chickens.

Bacteria previously classified as “*Actinobacillus salpingitidis*,” *Pasteurella haemolytica*-like, or *Pasteurella anatis* have recently been reclassified and relocated into a new genus, *Gallibacterium*, of the family *Pasteurellaceae* Pohl 1981 (12). At present, the genus includes the species *Gallibacterium anatis* and *Gallibacterium* genomospecies 1 and 2. *G. anatis* can be separated from other *Gallibacterium* spp. by phenotypic characterization, whereas *Gallibacterium* genomospecies 1 and 2, as the names imply, can be identified only by genotypic methods due to the phenotypic heterogeneity that characterize these species (12).

The nature of *Gallibacterium* infections is poorly understood. It has been suggested that the bacteria constitute a part of the normal flora of the upper respiratory tract as well as the lower genital tract of chickens (5, 27, 28). A recent investigation with clinically healthy chickens from different Danish layer production systems showed that hemolytic *Gallibacterium* was highly prevalent in birds from production systems with moderate or low levels of biosecurity (8). Mirle et al. (24) examined 496 hens with reproductive tract lesions and isolated *Gallibacterium* in pure culture from 23% of the diseased organs, whereas the second most prevalent agent, *Escherichia coli*, was isolated from 21% of the cases. In addition, others have isolated *Gallibacterium* in pure cultures of samples from animals with various pathological lesions or conditions, including salpingitis, oophoritis, peritonitis, pericarditis, hepatitis, enteritis, upper respiratory tract lesions, and septicemia (1, 6, 18–20, 27, 28, 31–34). Evidently, the bacteria are capable of causing serious, systemic infections affecting multiple organ systems, but the mechanisms of pathogenesis remain obscure.

The *Gallibacterium*-induced lesions that have been reported are not pathognomonic, and detection and identification are dependent on classical isolation and identification procedures, including phenotypic characterization (12). As is the case for most other genera of the family *Pasteurellaceae* Pohl 1981, the genus *Gallibacterium* is phenotypically a heterogeneous group (12, 30). Phenotypic characterization therefore involves laborious and time-consuming methods, which may also give ambiguous results due to variable outcomes. This in turn leads to difficulty in interpretation of the genus and species designations from some earlier studies, in which only a relatively few phenotypic characters have been investigated (7). Additionally, *Gallibacterium* may be underestimated as a cause of salpingitis and/or peritonitis due to the aforementioned limitations of the diagnostic methods available.

Establishment of an alternative, more accurate, and reliable detection method is warranted. Consequently, the aim of the present study was to develop a genotypic identification method allowing specific detection and evaluation of the spatial distribution of *Gallibacterium* in the host.

Rapid and specific identification of individual bacterial cells can be achieved by the fluorescent in situ hybridization technique (FISH) (15), which is based on fluorescent material-labeled oligonucleotides complementary to bacterial 16S rRNA. This poses advantages compared with traditional culture-based methods, as it is not restricted to live or intact cells, making it suitable for the detection of viable nonculturable cells and other fastidious organisms difficult to culture outside their natural habitat. FISH has been applied to the sensitive detection of microorganisms in situ and has been used to reveal bacterium-host interactions at the cellular level (4). The method has proved to be a valuable tool in elucidating the pathogenesis and spatial distribution of a range of different bacteria, including *Pasteurella multocida* in chicken and por-

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TABLE 1. Bacterial taxa included in GAN850 probe specificity testing with whole cells

Bacterial strain ^a	Bacterial taxon	Animal source	Hybridization signal ^b
NCTC11413 ^T	<i>G. anatis</i> (<i>P. anatis</i>)	Duck	+
CCM5976	<i>Gallibacterium</i> genomospecies 2 (<i>A. salpingitidis</i>)	Chicken	+
34346 Ovary	<i>G. anatis</i> (<i>A. salpingitidis</i>)	Chicken	+
10672-9	<i>G. anatis</i> (<i>A. salpingitidis</i>)	Chicken	+
12656-12 Liver	<i>G. anatis</i> (<i>A. salpingitidis</i>)	Chicken	+
24K10	<i>G. anatis</i> (<i>A. salpingitidis</i>)	Chicken	+
CCM5975	<i>G. anatis</i> (<i>A. salpingitidis</i>)	Chicken	+
Hom 69	<i>G. anatis</i> (<i>A. salpingitidis</i>)	Chicken	+
CCM5974	<i>Gallibacterium</i> genomospecies 1 (<i>A. salpingitidis</i>)	Chicken	+
NCTC4189 ^T	<i>Actinobacillus lignieresii</i>	Cow	—
ATCC 29546 ^T	<i>Pasteurella avium</i>	Chicken	—
NCTC11296 ^T	<i>Pasteurella gallinarum</i>	Chicken	—
NCTC10322 ^T	<i>P. multocida</i> subsp. <i>multocida</i>	Pig	—
NCTC10204 ^T	<i>P. multocida</i> subsp. <i>gallicida</i>	Bovine	—
NCTC11411 ^T	<i>Pasteurella langaa</i>	Chicken	—
CCUG3713 ^T	<i>Pasteurella volantium</i>	Fowl	—
CCUG18782	<i>Pasteurella</i> sp. strain A	Chicken	—
NCTC11414	Bisgaard taxon 2	Duck	—
NCTC11412	Bisgaard taxon 3	Duck	—
B7/99/1	Bisgaard taxon 3 biovar 1	Budgerigar	—
S/C1101	Bisgaard taxon 14 biovar 1	Turkey	—
F75	Bisgaard taxon 22	Chicken	—
F66	Bisgaard taxon 26 biovar 1	Duck	—
F97	Bisgaard taxon 26 biovar 2	Duck	—
HPA106	Bisgaard taxon 32	Sparrow hawk	—
101	Bisgaard taxon 33	Parrot	—
B301529/00/1	Bisgaard taxon 40	Gull	—
SA4461	<i>Haemophilus paragallinarum</i> biovar 2	Chicken	—
4237/2sv	<i>Riemerella anatipestifer</i>	Chicken	—
G9	<i>Salmonella enterica</i> serotype Gallinarum	Chicken	—
14R525	<i>E. coli</i>	Human	—
726-82 ^T	<i>Coenonia anatina</i>	Duck	—
CCUG23171 ^T	<i>Ornithobacterium rhinotracheale</i>	Turkey	—

^a Superscript T, type strain.^b +, a strong hybridization signal; —, no signal.

cine tissues (22), *Haemophilus somnus* in bovine lung tissue (35), and *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in porcine intestinal tissue (10).

In this study, we describe a detection method based on a cyanine dye 3.18 (Cy3)-labeled oligonucleotide probe specific

for *Gallibacterium*. The specificity of the probe was confirmed by the recovery of negative hybridization signals with 25 other poultry-associated bacterial species, including members of the families *Pasteurellaceae*, *Enterobacteriaceae*, and *Flavobacteriaceae*. Furthermore, the probe was shown to be specific and to

TABLE 2. Alignment of probe GAN850 sequence and 16S rRNA sequences from related bacterial taxa in the family *Pasteurellaceae*

Bacterial species	16S rRNA sequence ^a	GenBank accession no.	Reference
Probe GAN850	3'-CATACCGAGAGCTTCGTT-5'		This study
Non-probe GAN850	5'-GTATGGCTCTCGAAGCAA-3'		This study
<i>G. anatis</i> (<i>P. anatis</i>)	5'-.....-3'	AF228001	12
<i>Gallibacterium</i> genomospecies 1 (<i>A. salpingitidis</i>)	C-.....	AF228015	12
Bisgaard taxon 3 biovar 1	. . T . . TC	AY172724	13
Bisgaard taxon 32	-TC T .	AY172729	13
Bisgaard taxon 2	GAT . . TC T .	L06078	17
Bisgaard taxon 3	ACT . . TC T .	L06079	17
Bisgaard taxon 26	TC- . . TGCC T .	AF224284	30
<i>P. multocida</i> subsp. <i>multocida</i>	C- . . . GCCC T .	AF294410	21
<i>P. langaa</i>	C- GCCC . T . . . T .	M75053	17
<i>A. lignieresii</i>	TC- . . TGCCC T .	M75068	17
<i>P. avium</i>	C- . . . TGCCC . T . . . T .	M75058	17
<i>P. gallinarum</i>	C- . . . TGCCC . T . . . T .	M75059	17
<i>P. volantium</i>	C- . . . TGCCC . T . . . T .	M75070	17
<i>Pasteurella</i> sp. strain A	GG . . . C . CGTA . CTA . TT	M75055	17

^a Dot, base similarity to the complementary probe sequence; hyphen, a deletion in the sequence.

FIG. 1. FISH and histopathology demonstrating the ability of probe GAN850 to hybridize with *G. anatis* in pure culture and in infected formalin-fixed tissues. The scale bars indicate the magnifications of the images. (A) Pure culture of *G. anatis* strain 12656-12 showing a bright signal with GAN850; (B) pure culture of *Coenonia anatina* demonstrating a clear signal with EUB338; (C) splenic tissue from a chicken infected intravenously with *Gallibacterium* showing basophilic aggregates of bacteria in the ellipsoids (thin black arrow) surrounding the penicillium capillaries (thick black arrow) (HE stain); (D) splenic tissue from a chicken infected intravenously with *Gallibacterium* and hybridized with GAN850 yielding a bright signal (arrow) in patterns corresponding to the basophilic aggregates observed in panel C; (E) liver tissue from a chicken infected intraperitoneally with *Gallibacterium* and hybridized with GAN850 showing single cells of *G. anatis* positioned on the serosal side of the liver (arrow); (F) as for panel E, but hybridized with non-GAN850 and showing no hybridization signal.

be able to resolve the spatial distribution of *Gallibacterium* spp. in spleen and liver tissues from experimentally infected chickens.

MATERIALS AND METHODS

Bacterial strains and media. Thirty-three bacterial strains were included in the study (Table 1). The bacteria were stored at -80°C and cultivated overnight on

blood agar base (Oxoid, Hampshire, United Kingdom) with 5% citrated bovine blood. Single colonies were cultured in brain heart infusion broth (Oxoid) with shaking at 37°C . Bacterial concentrations and growth phase were estimated by determination of the optical density at 600 nm prior to fixation.

Fixation of bacterial cells. One hundred microliters of cultured bacterial cells in logarithmic growth phase was fixed in 800 μl of $1\times$ phosphate-buffered saline (PBS; Life Technologies, Taastrup, Denmark) with 4% paraformaldehyde

(Sigma Chemical Co., St. Louis, Mo.) and incubated for 1 h at 37°C. The bacteria were pelleted by centrifugation at $5,000 \times g$ for 5 min, washed in 500 μ l of 1× PBS with 0.1% (vol/vol) Nonidet P-40 (Sigma), and resuspended in a 1:1 mixture of storage buffer (20 mM Tris-HCl [pH 7.5], 96% ethanol). The fixed cells were stored at -20°C until use.

Oligonucleotide probes. The 16S rRNA sequences from 14 taxa of the family *Pasteurellaceae* Pohl 1981 from GenBank were aligned by use of the Pileup program (Wisconsin Sequence Analysis Package; Genetics Computer Group, Madison, Wis.) (Table 2). A *Gallibacterium*-specific oligonucleotide probe, GAN850 (5'-TTGCTTCGAGAGCCATAC-3'), and its complementary probe, non-GAN850 (5'-GTATGGCTCTCGAAGCAA-3'), were selected for use in further experiments. The uniqueness of the probe sequence was confirmed by a search with the BLASTn program (2). A probe regarded to be specific for all members of the eubacterial domain except the orders *Planctomycetales* and *Verrucomicrobia* (14), EUB338, 5'-GCTGCCTCCGCTAGGAGT-3' (3), was included as a positive control. The genus- and eubacterium-specific probes were synthesized; monolabeled at the 5' end with Cy3 and cyanine dye 5.18 (Cy5), respectively; and purified by high-pressure liquid chromatography (TagC, Copenhagen, Denmark).

In situ hybridization of bacterial cells. Prior to hybridization, fixed bacterial cells were bound to poly-L-lysine (Sigma)- and Teflon-coated slides (Novakemi AB, Enskede, Sweden) and dehydrated by sequential washes in 70 and 96% ethanol (3 min each). Ten microliters of hybridization buffer (15% [vol/vol] formamide, 20 mM Tris [pH 7.0], 0.9 M NaCl, 0.1% [vol/vol] sodium dodecyl sulfate) and 5 ng of probe were applied to each well, followed by hybridization in a humidified chamber. The fluorescence signal intensity was evaluated for all strains hybridized with probes GAN850, non-GAN850, and EUB338 at 37°C; with probes GAN850 and EUB338 at 42°C; and with probe GAN850 at 47°C. The duration of the hybridization was at least 1 h. The slides were then washed in hybridization buffer in a Coplin jar for 10 min at the hybridization temperature and were subsequently transferred to a washing buffer containing 20% (vol/vol) formamide for 10 min at the hybridization temperature. The slides were finally rinsed in MilliQ-water and air dried in the dark. All hybridizations were carried out at least twice.

Preparation of histological sections for hybridization. Liver and spleen tissue originated from chickens experimentally infected with *G. anatis* strain 12656-12 as described previously (9). Briefly, 15-week-old chickens were inoculated with *G. anatis* by the intravenous or intraperitoneal route. Chickens inoculated with saline were included as controls. Both heterophil-depleted chickens (which were treated with 5-fluorouracil to promote *Gallibacterium* invasion in the chickens) and chickens with normal immune status (which were treated with saline) were infected with 10^7 CFU. The chickens were killed; and tissue specimens were collected at 3, 12, and 24 h postinoculation. The tissue was immediately transferred to 10% phosphate-buffered formalin for fixation for 24 h, followed by dehydration and embedment in paraffin prior to preparation of 3- to 4- μ m-thick cross sections. The sections were mounted on adhesive slides (Super Frost/Plus; Menzel-Gläser, Braunschweig, Germany).

In situ hybridization of tissue sections. Tissue sections were deparaffinized with xylene, dehydrated twice with 99% ethanol for 3 min each time, and air dried. The hybridization and washing steps were performed essentially as described above for the bacterial samples except for the addition of 100 μ l of hybridization buffer and 200 ng of probe per tissue section. Histochemical staining of the splenic tissue was performed with hematoxylin-eosin (HE) stain for histopathological interpretation. A coverslip was mounted on hybridized slides by application of a few drops of nonfluorescent paraffin oil on the tissue sections.

Microscopy and image analysis. The slides were examined with a 15 or 26 filter (Carl Zeiss, Oberkochen, Germany) for visualization of Cy3 and Cy5, respectively, with an Axioplan II epifluorescence microscope (Zeiss) equipped for epifluorescence with a 100-W mercury lamp. A fluorescein isothiocyanate-Cy3 double filter (Chroma Technology Corp., Brattleboro, Vt.) was used when background staining of tissue as well as fluorescence from probe GAN850 was desired in order to ascertain the spatial distributions of the bacterial cells. Tissue sections stained with HE stain were examined by light microscopy. Images were acquired with a Zeiss AxioCam digital camera.

RESULTS

Specificity of probe sequence and optimization of hybridization conditions. A region corresponding to base positions 847 to 864 in the *E. coli* 16S rRNA gene sequence where at least three mismatches separated *Gallibacterium* from 12 closely

related members of the family *Pasteurellaceae* was identified (Table 2). A search of the GenBank database with the BLASTn program and the *Gallibacterium*-specific sequence did not show a high degree of similarity to the sequences of other non-*Gallibacterium* species, confirming the uniqueness of the probe.

The specificity of probe GAN850 was tested with pure cultures of related organisms which could pose a differential diagnostic problem. A strong fluorescent signal was detected from the *Gallibacterium* spp. (Fig. 1A), whereas no or minimal detectable signals were obtained from all 24 non-*Gallibacterium* strains included (Table 1). The reverse and complementary probe, non-GAN850, did not show any signal upon hybridization to any of the strains included (data not shown). Hybridization of the eubacterial probe, EUB338, with all the strains included showed a signal intensity corresponding to the level observed with GAN850 hybridized with members of the genus *Gallibacterium* (Fig. 1B).

The signal intensity became slightly weaker when the hybridization temperature was raised from 37 to 42°C and to 47°C but did not change the overall specificity of probe GAN850 (data not shown). Alteration of the hybridization time between 1 and 24 h did not influence the hybridization specificity or the signal intensity of GAN850.

Histological findings and tissue hybridization with fluorescent probes. HE staining of splenic tissue from chickens inoculated intravenously showed basophilic microcolonies and bacterial aggregates, primarily in the ellipsoids surrounding the penicilliform capillaries (Fig. 1C). Necrotic splenic cells and eosinophilic material were also identified in the ellipsoids. Spleen tissues from noninfected controls had no detectable lesions (data not shown).

A strong hybridization signal was obtained with probe GAN850 in tissue sections from infected chickens. The signal distribution corresponded to the microcolonies and basophilic aggregates of bacteria seen in the ellipsoids in the HE-stained spleen sections (Fig. 1D). Single bacteria could be visualized by HE staining and GAN850 hybridization (Fig. 1E) in the liver tissue sections from the chickens inoculated intraperitoneally, in which bacteria were apparent in the exudates covering the serosal surfaces of the livers. Tissue hybridization with the eubacterial probe, EUB338, always revealed a pattern similar to that obtained with GAN850 with infected chicken tissue (data not shown), whereas hybridization with the complementary probe, non-GAN850, was negative for infected as well as noninfected control tissues (Fig. 1F).

DISCUSSION

To our knowledge, only the FISH procedure developed in the present study allows specific identification of the recently defined genus *Gallibacterium* in both culture and tissue samples. This is of particular importance, as detection and identification of these bacteria remain problematic due to the ambiguity of the phenotypic interpretations associated with difficulties in differentiating *Gallibacterium* from other bacterial genera on the basis of phenotypic methods (12).

FISH-based methods have been demonstrated to be applicable to the identification of bacteria in their natural environment (10, 25, 26). Moreover, a recent study by Tegtmeier et al.

(35) demonstrated that bacterial cultivation is the least sensitive method for the detection of bacteria in tissue in comparison to in situ hybridization, immunohistochemistry, and PCR, underlining the importance and necessity of alternatives to traditional detection methods. *Gallibacterium* spp. have the typical characteristics of an opportunistic pathogen, relying on predisposing factors, such as coinfections of viral, bacterial, or parasitic origin, stress, or hormonal imbalances, to elicit disease (18, 31). Identification of *Gallibacterium* spp. in mixed bacterial populations by a non-culture-dependent identification method would be advantageous and avoid selection of certain bacterial species which could distort interpretation of the actual species composition in the sample investigated (11). This is further underlined by the fact that certain isolates of hemolytic *Gallibacterium* grow only under microaerophilic conditions. We did not test the method with naturally infected chickens, in which profound histological changes and the presence of a mixed bacterial population may lower the performance of the present technique. Previous studies have indicated that application of an in situ PCR detection method can improve the detection sensitivity compared to those of culture and FISH-based methods (16, 29). Although PCR-based methods are superior for detection, they are still less useful for evaluating the spatial distribution of bacterial cells within infected tissue, which plays an important role in elucidating the pathogenesis. Furthermore, the very bright signal observed from the experimentally infected tissue indicates that no major problems are likely to be expected with regard to the signal intensity from *G. anatis* in naturally infected tissue.

Minor variations in signal intensities were observed between repeated hybridizations with the same strain under identical hybridization conditions; however, the magnitudes of the variations were negligible, and a clear distinction between positive and negative signals was always possible.

Lack of specificity is commonly encountered in the development of a genus- or species-specific probe (25). Alignments of sequences with published 16S rRNA sequences did not identify any homologous matches. These results were further supported by specificity testing with probes GAN850 and EUB338 in parallel, confirming the accessibility and abundance of 16S rRNA in all the non-*Gallibacterium* species tested. These did not give signals upon hybridization with GAN850, but clearly detectable signals were seen with EUB338. GAN850 was also specific when hybridizations were carried out with chicken tissue. We did not observe unspecific binding, cross hybridization, or autofluorescence at a magnitude that disturbed the signal from *Gallibacterium*. It has been shown that these often hamper visualization of the hybridization signals in tissue (23). The oligonucleotides labeled with Cy3 and Cy5 used in the present study gave a clear bright signal, which enabled the use of narrow-band-pass filters, enhancing the signal-to-noise ratio considerably compared to those obtained with classical fluorophores, such as fluorescein and rhodamine derivatives, confirming the observations of Wessendorf and Brelje (36). However, some counterstaining can be useful for visualization of the spatial distribution of bacteria within tissue (10, 22), which in the present study was achieved by the use of a double filter set. The double filter set allowed the passage of signals from the labeling dye as well as specified wavelengths of autofluorescence from the host tissue.

In conclusion, we have designed an oligonucleotide probe targeting 16S rRNA which enables the specific detection of *Gallibacterium* species in situ. This genotypic diagnostic method for *Gallibacterium* detection represents the first alternative to the present phenotype-based method to be described. A particular advantage is its ability to identify *Gallibacterium* spp. in infected tissues, which can be of major importance at elucidating the pathogenesis and the bacterium-host cell interactions of this poorly understood microorganism.

ACKNOWLEDGMENT

The Danish Agricultural and Veterinary Research Council financed this work (grant 9702797).

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